A novel *Sry*-downstream cellular event which preserves the readily available energy source of glycogen in mouse sex differentiation

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Summary

Sry is transiently activated in pre-Sertoli cells of the gonadal ridge to initiate testis differentiation in mice. In pre-Sertoli cells, however, the cellular events induced immediately after the onset of _Sry_ expression remain largely unknown. Here we show that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of _Sry_ action. In developing XY gonads, glycogen accumulation starts to occur in pre-Sertoli cells from around 11.15 dpc (tail somite 14 stage) in a center-to-pole pattern similar to the initial _Sry_ expression profile. Glycogen accumulation was also found in XX male gonads of _Sry_-transgenic embryos, but not in XX female gonads of wildtype embryos at any developmental stage. In vitro analyses using various culture conditions suggest that testis-specific glycogen deposition is a tissue-autonomous event that can be induced even in serum-free conditions and in a culture of gonadal explants without adjacent mesonephros. Moreover, glycogen accumulation in pre-Sertoli cells was significantly inhibited in vitro by the PI3K inhibitor LY294002, but not by the MEK inhibitor PD98059. Active phospho-AKT (PI3K effector) showed a high degree of accumulation in gonadal somatic cells of genital ridges in a testis-specific manner, both in vitro and in vivo. Therefore, these findings suggest that immediately after the onset of _Sry_ expression, activation of the PI3K-AKT pathway promotes testis-specific glycogen storage in pre-Sertoli cells. To the best of our knowledge, this is a novel _Sry_-downstream cellular event which preserves this readily available energy source in Sertoli cells for testis-specific morphogenesis and hormone production.

Supplementary material available online at
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Key words: *Sry*, *Sox9*, Glycogen, Sertoli cell, PI3K-AKT signaling, Sex differentiation

Introduction

Sry (Sex-determining region of the Y) is essential for initiating male sex differentiation in mammals (Sinclair et al., 1990; Koopman et al., 1991). *Sry* is transiently expressed in a center-to-pole wave along the anteroposterior (AP) axis of the XY gonad for a very short period just before testis formation in mice (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). Thus, *Sry* is likely to act as a trigger for a cascade of molecular and cellular events that direct the bipotential gonad to differentiate into a testis in a center-to-pole manner. *Sox9*, a *Sry*-related gene, is one of the candidate genes directly upregulated by *Sry*. Shortly after the onset of *Sry* expression, *Sox9* is also upregulated in a center-to-pole pattern similar to that of the initial *Sry* expression profile (Morais da Silva et al., 1996; Kent et al., 1996; Schepers et al., 2003). Human *SOX9* mutation causes XY sex reversal in most cases (Foster et al., 1994; Wagner et al., 1994). Homozygous deletion of *Sox9* in mouse XY gonads interferes with testis differentiation and activation of testis-specific markers *Mis* and *P450scc* (Chaboissier et al., 2004). Moreover, miss-expression of *Sox9* in mouse XX gonads results in testis development, as demonstrated by the findings from two transgenic lines (Bishop et al., 2000; Vidal et al., 2001). Therefore, these reports suggest that *Sry* directly or indirectly promotes testis-specific *Sox9* activation, and that *Sox9* is involved in the initiation and maintenance of Sertoli cell differentiation during early phases of testis differentiation.

In mouse sex differentiation, three testis-specific cellular events: cell proliferation, cell migration and vasculogenesis, are known to direct early testiculogenesis. Increased proliferation of the coelomic epithelium of gonads occurs between 11.3 and 12.0 dpc (Schmahl et al., 2000; Schmahl and Capel, 2003). This proliferation may give rise to a certain population of pre-Sertoli cells early on and to interstitial cells throughout this period (Karl and Capel, 1998). The cells contributing to the interstitium, including vascular endothelial cells and peritubular myoid cells, migrate into the testis from the adjacent mesonephros (Buehr et al., 1993; Martineau et al., 1997; Capel et al., 1999; Brennan et al., 2002). These cells are also required for testicular cord formation (Martineau et al.,

1997; Tilmann and Capel, 1999). These early cellular events are likely to be controlled indirectly by *Sry* and/or *Sox9*, because both *Sry* and *Sox9* are expressed specifically in pre-Sertoli cell lineage, but not in the coelomic epithelium, endothelial cells or mesonephric cells (Morais da Silva et al., 1996; Kent et al., 1996; Bullejos and Koopman, 2001; Albrecht and Eicher, 2001; Moreno-Mendoza et al., 2004). Although pre-Sertoli cells have been shown to aggregate in the testicular cord at late phases of testis differentiation (from around 12.0 dpc), the cellular events induced immediately after *Sry* expression in pre-Sertoli cells remain largely unknown.

Testis-specific cellular events, such as proliferation, migration, vasculogenesis and cord morphogenesis, clearly indicate a difference in energy metabolism between male and female gonads during sex differentiation (Mittwoch, 2004). This further suggests that XY gonads require a higher energy metabolic rate for the dynamic morphogenesis of male gonads, compared with XX gonads, which exhibit no appreciable histological changes. Glucose, which plays a major role in energy metabolism, is stored as a readily available energy source, glycogen, in cells during various developmental and physiological states (Thong and Graham, 2002; Sinclair et al., 2003; Gruetter, 2003; Ferrer et al., 2003). Our previous study has shown that embryonic testis is one of the glycogen-rich tissues in mouse organogenic embryos, and that glycogen accumulation predominantly occurs in Sertoli cells within newly formed testicular cords at 12.5 dpc (Kanai et al., 1989). Moreover, the glycogen deposits in Sertoli cells rapidly disappear shortly after testicular cord formation, suggesting that the glycogen granules in Sertoli cells are involved as an energy source in the dynamic morphogenesis of the testis and active hormonal production at around 12.5 dpc. However, any further information including the timing of the onset of glycogen accumulation and its mechanism in developing XY gonads is not available at present.

In this study, we examined the spatiotemporal patterns of glycogen accumulation and its signaling pathways during early phases of mouse sex differentiation in order to analyze a possible mechanism of sex-dimorphic glycogenesis in mammalian sex differentiation. As a result, the present study is the first to show that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry*. We also demonstrated that such glycogen deposition is mediated via the PI3K/AKT signaling pathway, which is activated in gonadal somatic cells in a testis-specific manner. Therefore, these findings clearly illustrate that immediately after the onset of *Sry* expression, activation of the PI3K-AKT pathway may promote testis-specific glycogen accumulation in pre-Sertoli cells. This will shed light on a potential link between *Sry* action and sex-dimorphic energy metabolism in gonadal sex differentiation of mammals.

Materials and Methods

Animals and busulfan treatment

Embryos were obtained from pregnant female mice (ICR strain) from 10.5 to 12.5 dpc [7-30 tail somite (ts)]. In some cases, the XX sex-reversal transgenic line carrying the *Sry* transgene (Kidokoro et al., 2005) was also used in this study. After counting the tail somite number and separating the head tissues in each embryo for sex determination, genital ridges (i.e. gonad plus mesonephros) were used for the experiments described below. For busulfan treatment, busulfan (40 mg/kg body weight) was injected intraperitoneally into pregnant female mice at 9.5 dpc (McClive et al., 2003), and then the embryos were isolated at 11.5 and 12.5 dpc. In addition, genomic DNAs were prepared from the head region of each embryo, and the sex of each embryo was determined by PCR using *Zfy*-specific primers as described previously (Bowles et al., 1999).

Organ culture

To examine the effects of culture conditions and inhibitors for signaling molecules on glycogen accumulation in developing XY gonads, we performed organ cultures using genital ridges isolated at 9-14 ts. One of each pair of genital ridges was cultured in a medium containing an inhibitor, while the other was used as a control. The PI3K (phosphoinositide 3-kinase) inhibitor LY294002 (15 μM; Calbiochem) and the MEK (mitogen-activated or extracellular signal-regulated protein kinase) inhibitor PD98059 (50 μM; Calbiochem) were mainly used in this study. To analyze a possible contribution of the adjacent mesonephros on glycogen accumulation in XY gonads, the genital ridges were isolated at 14 ts, and the gonads were separated from the mesonephros under a dissecting microscope using a sharp needle. All explants were placed onto an ISOPORE membrane filter (3.0 μm; Millipore), floated on DMEM (Dulbecco's Modified Eagle's Medium; Sigma) containing 10% horse serum and penicillin/streptomycin (Gibco-BRL), and cultured at 37°C for 6 to 48 hours as described previously (Kanai et al., 1992; Hiramatsu et al., 2003). Some genital ridges were also cultured with DMEM alone (i.e. without 10% horse serum). All cultured explants were used for the following histochemical and immunoblot analyses.

Histological and histochemical analyses

For periodic acid Schiff (PAS) reaction, the isolated embryos were fixed in 10% formaldehyde containing 2% Ca $(CH_3COO)_2$ at 4°C for 12 hours, and then dehydrated in ethanol, and embedded in paraffin. Serial sagittal sections (approximately 5 μm in thickness) were cut and stained with PAS reagent (Kanai et al., 1989). To clarify the histochemical specificity for glycogen, two serial sections were pretreated with or without 0.1% α-amylase (Sigma) at 37°C for 1 hour before PAS staining. All gonadal images of serial sagittal sections (five embryos at 14 ts; four embryos each at 15 and 16 ts) were photographed. After the five regions along the anteroposterior (AP) axis (regions I-V in Fig. 4A) were selected in each gonadal image, we calculated the number of PAS-positive cells located in each region and those located adjacent to germ cells. Moreover, each gonadal area (regions I-V) was separately measured using the Scion image program (Ver. 4.02). In each embryo, we estimated (1) the total number of PAS positive cells per gonad, (2) the total number of PAS-positive cells located in each region, (3) the relative number of PAS-positive cells per unit area (cell number per mm^2) in each region, and (4) the ratio of PAS-positive cells located adjacent to germ cells.

For transmission electron microscopy, the genital ridges at 14-18 ts were fixed in 3% glutaraldehyde-0.05 M phosphate buffer (PB) containing 0.05 M sucrose at 4°C for 4 hours. After washing with 0.05 M PB containing 0.05 M potassium ferricyanide, the samples were postfixed in 1% OsO_4-0.05 M potassium ferricyanide in 0.05 M PB at 4°C for 2 hours. The specimens were then dehydrated in ethanol, and embedded in Araldite M. Ultrathin sections were cut using an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and then observed under a JEM 1010 transmission electron microscope at 80 kV.

Immunohistochemistry

For immunohistochemistry, both embryos and cultured explants were fixed in 4% PFA-PBS at 4°C for 4 hours, dehydrated, and then

embedded in paraffin. Deparaffinized sections were subjected to immunohistochemistry, using anti-SOX9 antibody (Ab) (10 ng/μl; kindly provided by Peter Koopman, University of Queensland, Australia) (Kent et al., 1996), anti-SF-1/Ad4BP Ab (1/1000 dilution; kindly provided by Ken-ichirou Morohashi, the National Institute for Basic Biology, Japan) (Hatano et al., 1994; Ikeda et al., 2001), and anti-phospho-AKT Ab (Ser473; 1/50 dilution; Cell Signaling Technology). The sections were incubated with each rabbit primary antibody at 4°C for 12 hours. For comparative staining with PAS reagent and anti-SOX9 or anti-SF-1/Ad4BP Ab, two consecutive 4 μm-thick mirror sections (i.e. two serial sections with the same cutting plane were put on separate slide glasses) were prepared from paraffin blocks of 4%-PFA-fixed samples. One section was stained using PAS, while another mirror section was incubated with anti-SOX9 or anti-SF-1/Ad4BP Ab at 4°C for 12 hours. Thereafter, the immunoreaction with each first antibody was visualized by HRP-labelled anti-rabbit IgG Ab using a Tyramide Signal Amplification kit (NEN Life Science Product). In addition, non-specific reactions could not be detected in germ cells or gonadal somatic cells when the sections were incubated with control rabbit IgG instead of anti-SOX9, anti-SF-1/Ad4BP or anti-phospho-AKT Ab.

Immunoblot analysis

The genital ridges at 9-11 ts were cultured at 37°C for 24 hours in 10% horse serum-DMEM with or without inhibitors. Then the explants were dissolved in non-reduced SDS sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue). After each protein sample was electrophoresed and transferred to nylon membranes, the blots were incubated with anti-phospho-AKT (1:1000 dilution), anti-AKT (1:1000 dilution; Cell Signaling Technology), anti-phospho-ERK (Thr202/Tyr204; 1:1000 dilution; Cell Signaling Technology) or anti-ERK (1:1000 dilution; Cell Signaling Technology) Ab at 4°C for 12 hours. The immunoreaction was visualized with HRP-labelled goat anti-rabbit secondary Ab using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) as described previously (Kanai et al., 1996).

Results

Testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* action

Our previous study showed that embryonic testis is a glycogen-rich tissue in mouse embryogenesis, and that glycogen accumulation predominantly occurs in Sertoli cells within newly formed testicular cords (Kanai et al., 1989) (Fig. 5C in this study). Fig. 1 shows a sex-dimorphic distribution of glycogen deposits in mouse embryos at 11.5 dpc [18 tail somite (ts) stage] using periodic acid Schiff (PAS) staining. PAS-positive reactions were found in skeletal muscles, notochord and the area surrounding the dorsal aorta in both XY and XX embryos at similar levels. In the gonadal region, however, PAS-positive reactions were detected in XY, but not XX, embryos (Fig. 1A-D). These reactions were predominantly observed in gonadal somatic cells near germ cells, i.e. presumptive pre-Sertoli cells (Fig. 1D). Such reactions completely disappeared when pre-incubated with α-amylase (Fig. 1G,H). Electron microscopic observations showed that glycogen granules were frequently found in the cytoplasm of gonadal somatic cells closely associated with germ cells in XY, but not XX, gonads (Fig. 1E,F), indicating that testis-specific glycogen accumulation occurs at least from 11.5 dpc (18 ts).

To examine the timing of the onset of glycogen deposition

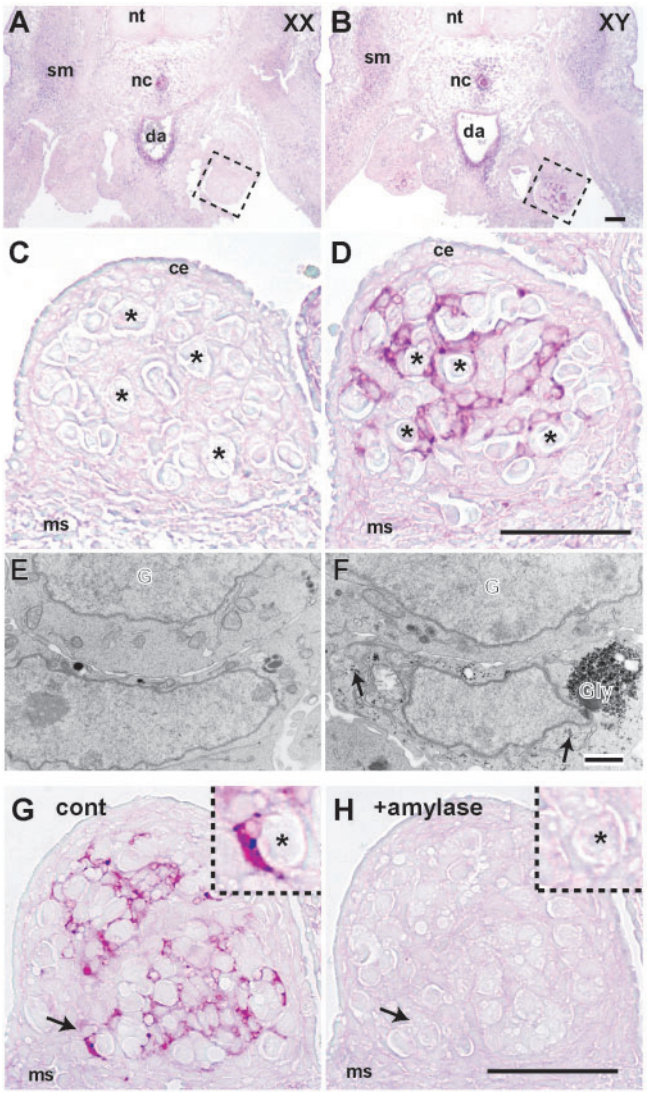

Fig. 1. A sex-dimorphic distribution of the glycogen deposits in the gonads at 11.5 dpc (18 ts stage). (A-D) Transverse sections of 11.5-dpc XX (A,C) and XY (B,D) embryos. Periodic acid Schiff (PAS) staining (red staining). In both XY and XX embryos, several tissues including notochord (nc), skeletal muscle (sm) and the area close to dorsal aorta (da) show positive PAS staining. However, in developing genital ridges, PAS reactions are observed only in the gonadal region of XY, but not XX, embryos. In the XY gonad, PAS-positive cells are located close to germ cells (asterisks) (D), whereas no positive cells are detected in XX gonad (C). Plates C and D show higher magnification images, indicated by the broken rectangle in plates A and B, respectively. asterisk, germ cell; ce, coelomic epithelium; da; dorsal aorta; ms, mesonephros; nc, notochord; nt, neural tube; sm, skeletal muscle. Bar, 50 μm. (E,F) Transmission electron micrographs showing an accumulation of glycogen granules (arrows; a massive glycogen deposit is indicated by 'Gly') in the cytoplasm of gonadal somatic cells closely associated with germ cells in the XY (F), but not the XX, gonad (E). G, germ cell; Gly, glycogen granule. Bar, 1 μm. (G,H) Two serial sections of the 11.5-dpc XY gonad were pretreated with (H) or without α-amylase (G) before PAS staining. α-amylase digestion results in a complete loss of PAS reaction in the XY gonad. The insets show higher magnification of the cells indicated by arrows. asterisk, germ cell; ms, mesonephros. Bar, 50 μm.

in developing XY gonads, we compared the spatial patterns of PAS reactions and immunostaining against SOX9 protein, one of the first factors induced by *Sry*, during early phases of mouse testis differentiation (Fig. 2A). SOX9-positive cells were first detected in the XY gonad isolated at 14 ts, and then increased in number at later stages (from 16 to 18 ts). Similar to the temporal pattern of SOX9 expression, PAS-positive cells were first detected in the XY gonad isolated at 14 ts, and then rapidly increased in number to aggregate into cord-like structures at 18 ts. In addition to XY gonads, PAS reactions were also observed in gonads isolated from XX male embryos carrying the *Sry* transgene (Fig. 2B, right panel). This is clearly in contrast to the absence of PAS-positive cells in wildtype XX gonads at all stages examined (Fig. 2A,B, left panels). Moreover, we separately performed the PAS reaction and immunohistochemical staining with anti-SF-1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig cells) or anti-SOX9 (pre-Sertoli cell marker) Ab using two consecutive mirror sections of the same PFA-fixed specimens. A comparative analysis of PAS reaction and anti-SF-1/Ad4BP staining revealed that PAS-positive cells clearly expressed SF-1/Ad4BP in their nucleus (Fig. 3A,B), although PAS reaction was markedly reduced in PFA-fixed gonads compared with those fixed with 10% formalin/2% $Ca(CH_3COO)_2$ (probably due to reduced preservation of glycogen in PFA-fixed samples). Moreover, in XY gonads, the nucleus of PAS-positive cells was positive for anti-SOX9 staining (when their nucleus was in the section; Fig. 3C,D). Because *Sry* expression is first detected in XY gonads at around 12 ts (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001), these findings clearly indicate that glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* during early phases of testis differentiation.

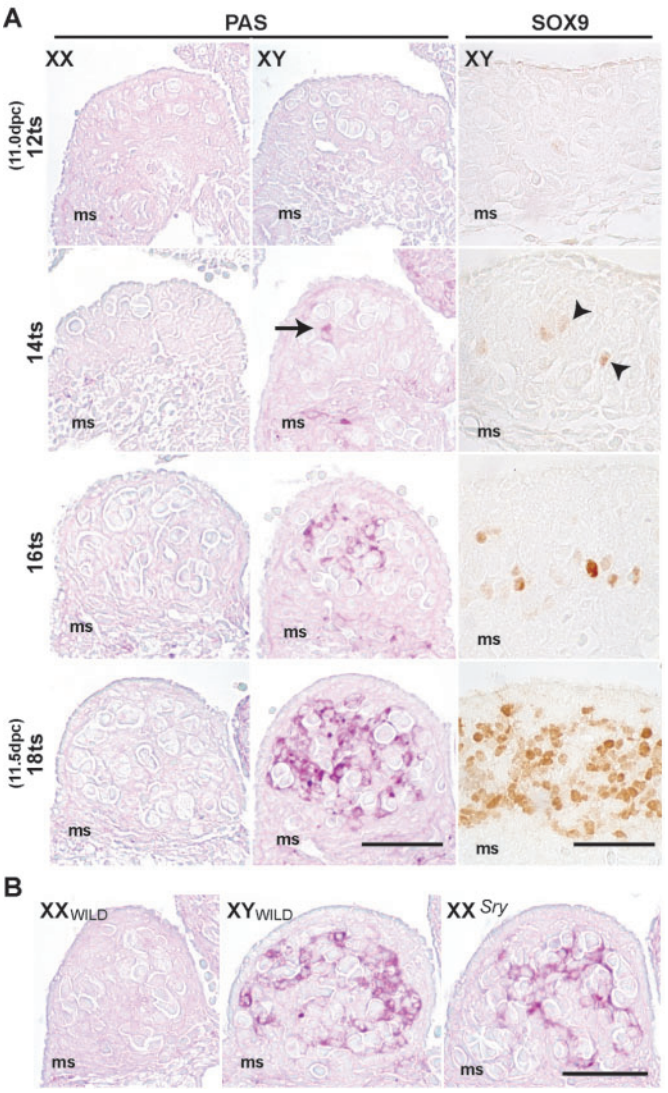

Fig. 2. Timing of the onset of glycogen accumulation in developing XY gonads and its accumulation in the 11.5-dpc XX male gonad of sex-reversal *Sry* transgenic mice. (A) Temporal patterns of PAS reactions (red staining; transverse sections) and immunostaining against SOX9 protein (brown staining; sagittal sections) in the XX (left) and XY (middle and right) gonads during early phases of sex differentiation. Similar to the temporal pattern of SOX9 expression, PAS-positive cells are first detected in the XY gonad isolated at 14 ts (arrow for PAS; arrowheads for SOX9). These cells rapidly increased in number, and then aggregated into cord-like structures at 18 ts. No PAS-positive cells are detected in XX gonads at any stages examined. Embryos at approximately 11.0 and 11.5 dpc show 12 and 18 ts, respectively. (B) Transverse sections of the 11.5 dpc (18 ts) embryos of XX wildtype, XY wildtype and XX *Sry* trangenic embryos stained with PAS. PAS reactions are seen in XY wildtype gonads, as well as in the XX gonads of male embryos carrying the *Sry* transgene. ms, mesonephros. Bar, 50 μm.

A center-to-pole pattern of glycogen accumulation in developing XY gonads

Previous studies have demonstrated that the expression of both *Sry* and *Sox9* is first detected in the central region of the XY gonad before their expression domains expand to both anterior and posterior ends during early phases of testis differentiation (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001; Schepers et al., 2003). In order to examine the spatial pattern of glycogen accumulation along the AP axis of developing XY gonads, consecutive sagittal sections spanning from the lateral to medial edges were stained with PAS reaction, and the number of PAS-positive cells in each gonadal region (regions I-V, equally divided along the AP axis of the gonad; left plates of Fig. 4A) was determined. The total number of PAS-positive cells per gonad increased rapidly from 14 to 16 ts [cell number per gonad±standard error: 4.0±0.9 at 14 ts (n=5); 26.3±8.7 at 15 ts (n=4); 419.5±43.3 at 16 ts (n=4)]. PAS-positive cells were found to be positioned predominantly in the middle regions (regions II to IV) at 14 ts (Fig. 4C; also see Fig. S1 in supplementary material). This center-restricted distribution expanded into the anterior (region I) and posterior (region V) edges from 15 to 16 ts.

Glycogen accumulation in pre-Sertoli cells is likely to be independent of germ cells

An interesting finding from the present histochemical observations is that PAS-positive cells were frequently found near germ cells in XY gonads even at 14-15 ts (right panels in Fig. 4A). Electron microscopic analysis of XY gonads at 15 ts also confirmed that glycogen-rich cells were found to be directly associated with germ cells (Fig. 4B). A quantitative

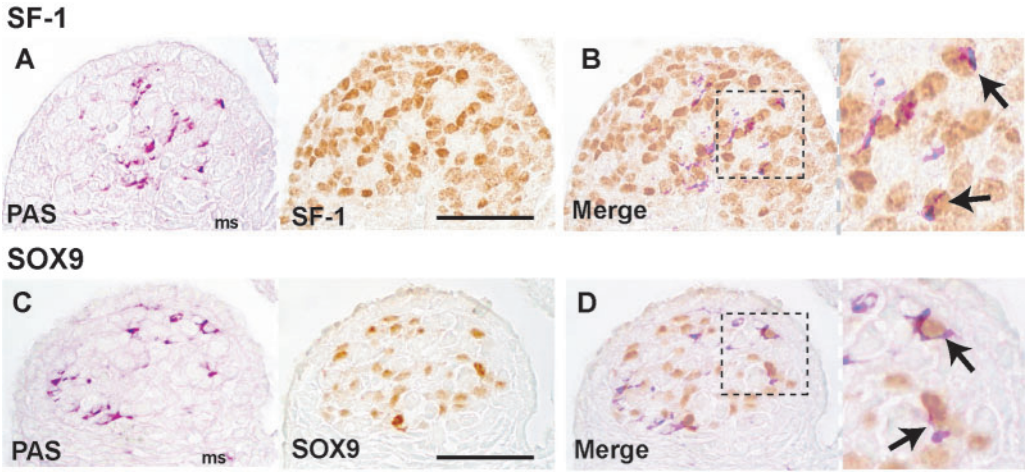

Fig. 3. PAS reaction and anti-SF-1/Ad4BP or anti-SOX9 staining of two consecutive mirror sections of 11.5-dpc XY gonads demonstrating that glycogen accumulation occurs in pre-Sertoli cell lineage. PAS reaction (red staining) and immunohistochemical staining (brown staining) with anti-SF-1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig cells; A,B) or anti-SOX9 (pre-Sertoli cell marker; C,D) Ab were performed using two consecutive mirror sections of the same PFA-fixed specimens. For comparison, images of immunostained mirror sections are computationally reversed. Plates B and D are the merged images of PAS reaction (left) and immunoreaction (right; reversed images) in plates A and C, respectively. Plates B and D also include higher magnification images (right), indicated by the boxed area in the corresponding left plate. PAS-positive cells clearly overlap with gonadal somatic cells expressing SF-1/Ad4BP (arrows in B) or SOX9 (arrows in D) in the XY gonads. ms, mesonephros. Bar, 50 μm.

analysis of PAS-stained sections revealed that, even at 14-15 ts, approximately 75% of PAS-positive cells were located in the area adjacent to germ cells at the sectioning level [ratio of PAS-positive cells closely associated with germ cells per total PAS-positive cells±standard error: 81.7±5.5% at 14 ts (n=5); 73.0±8.0% at 15 ts, 76.3±4.2% at 16 ts (n=4)]. In order to investigate a possible involvement of germ cells in glycogen accumulation in pre-Sertoli cells, we experimentally produced gonads with a severely reduced number of germ cells by administration of busulfan to 9.5-dpc pregnant mice (McClive et al., 2003), and examined the effects of germ cell loss on PAS-staining pattern in XY gonads at 11.5 (18 ts) and 12.5 (30 ts) dpc (Fig. 5). It was shown that there was no appreciable difference between busulfan-treated gonads and the control

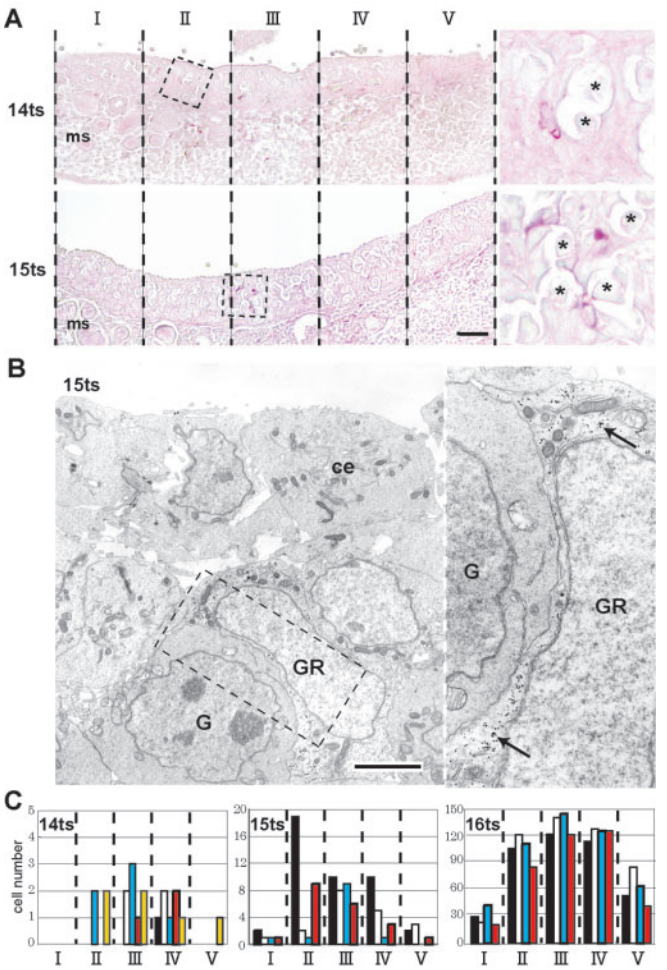

Fig. 4. A center-to-pole pattern of glycogen accumulation along anteroposterior (AP) axis of developing XY gonads and close association between glycogen-rich cells and germ cells during early phases of testis differentiation. (A) Sagittal sections of XY gonads isolated at 14 ts (upper) and 15 ts (lower) stages. PAS staining. Regions I-V are equally divided along the AP axis of the gonad. PAS-positive cells at 14 and 15 ts are located in regions II and III, respectively. Interestingly, they are frequently found in an area near germ cells even at 14 and 15 ts stages (right panel). Right panels show higher magnification images, indicated by the boxed area in the corresponding left panel. Asterisk, germ cell; ms, mesonephros. Bar, 50 μm. (B) Transmission electron micrographs showing a direct association between glycogen-positive cells and germ cells in XY gonad at 15 ts. Right panel shows a higher magnification image, indicated by the broken rectangle in left panel. Arrows indicate glycogen granules. ce, coelomic epithelial cells; G, germ cells; GR, glycogen-rich cells. Bar, 5 μm. (C) All consecutive sagittal sections were stained with PAS reaction, and then the total number of positive cells in each gonad was measured separately in regions I-V, which were equally divided along the AP axis of the gonad (broken lines in A). The vertical axis represents the PAS-positive cell number per gonad, whereas the horizontal axis represents regions I-V of the gonads. In each graph, bars of the same color show the cell number in each region of the same gonad (14 ts, five gonads; 15 and 16 ts, each four gonads). PAS-positive cells are positioned predominantly in the middle regions (regions II to IV) at 14 ts. This center-restricted distribution clearly expands into the anterior (region I) and posterior (region V) edges from 15 to 16 ts.

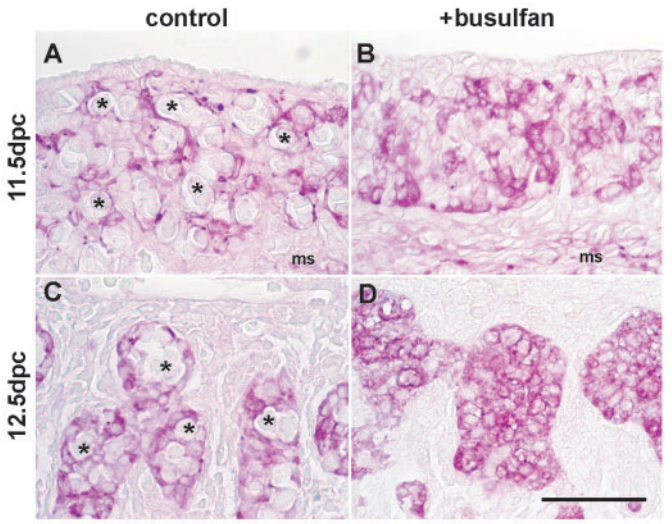

Fig. 5. Glycogen accumulation occurs in XY gonads with severely reduced germ cells that are experimentally induced by busulfan treatment. Sagittal sections showing the effects of germ cell loss on PAS-staining pattern in XY gonads at 11.5 (18 ts; upper plates) and 12.5 (30 ts; lower plates) dpc. PAS staining. No germ cells are detected in these sections of busulfan-treated XY gonads shown in plates B and D. In XY gonads treated with busulfan, PAS-positive cells are properly aggregated into slender cord-like structures (B) similar to those in control XY gonads at the same stage (A), despite the drastic reduction in number of germ cells. In the testes at 12.5 dpc, the testicular cords are formed in the differentiated testes without germ cells, and Sertoli cells show positive PAS staining in both control (C) and busulfan-treated (D) testes. asterisk, germ cell; ms, mesonephros. Bar, 50 μm.

either at 11.5 or 12.5 dpc. In short, in XY gonads treated with busulfan, PAS-positive cells were properly aggregated into slender cord-like structures similar to those in control XY gonads at the same stage, despite the drastic reduction in number of germ cells (Fig. 5A,B). In the testes at 12.5 dpc, the testicular cords were formed in the testes without germ cells and all Sertoli cells showed positive PAS staining (Fig. 5C,D).

Testis-specific glycogen deposition in genital ridge organ cultures: effects of developmental stage at culture initiation, serum withdrawal, and absence of adjacent mesonephros

In our previous study, 3-day cultures of XY gonadal explants initiated at stages prior to 11 ts (approximately 11.0 dpc) failed to induce either testis cord formation or testis-specific *Sox9* expression (Hiramatsu et al., 2003). This is in contrast to the proper induction of Leydig cell differentiation (Hiramatsu et al., 2003) and *Sry* expression (see Fig. S2 in supplementary material) in the cultured explants initiated at 9-11 ts. In order to examine the effect of gonadal stage at culture initiation on sex-dimorphic glycogen accumulation, we cultured genital ridges isolated at 9-10 and 12-13 ts in 10% horse serum-DMEM, and examined the time course of accumulation patterns of glycogen deposits in developing gonads in vitro (Fig. 6). In XY explants at 12-13 ts, PAS-positive cells were first detected at 6 hours, and the cells rapidly increased from 12 to 24 hours. No PAS-positive cells were detected in XX explants at 12-13 ts even after 48 hours, which suggests that the present culture condition is capable of inducing sex-dimorphic glycogen accumulation in developing gonads. Interestingly, in all cultures of genital ridges isolated at 9-10 ts, PAS-positive cells were observed in the gonadal region in a testis-specific manner, suggesting that glycogen accumulation in pre-Sertoli cells is not directly associated with subsequent testicular cord formation and Sertoli cell differentiation. Most interestingly, when we cultured 12 ts genital ridges in serum-free DMEM medium (i.e. without 10% horse serum), it was shown that glycogen deposition was induced in XY, but not in XX, explants, despite obvious growth defects in both XY and XX genital ridges (four out of four in each sex; Fig. 6).

It has been shown that *Sry*-dependent mesonephric cell migration is crucial for Sertoli cell differentiation and *Sox9* expression during early stages of mouse testis differentiation (Buehr et al., 1993; Martineau et al., 1997; Tilmann and Capel, 1999). In order to analyze a possible contribution of mesonephric cells to testis-specific glycogen accumulation, we next examined the PAS-staining pattern in culture explants with or without adjacent mesonephros isolated at 14 ts (Fig. 7). In 24-hour cultured gonads with adjacent mesonephros (i.e. genital ridge), glycogen accumulation was induced in all five XY explants, but not in any XX explants (Fig. 7B,E). Similar to the pattern of glycogen accumulation in the control cultures, PAS-positive cells were observed in all XY, but not in any XX, explants without mesonephros (four out of four in each sex; Fig. 7C,F). With regards to the present finding of testis-specific induction of glycogen accumulation even in serum-free cultures, this finding suggests that this sex-dimorphic glycogen deposition is a tissue-autonomous cellular event downstream of *Sry*.

A testis-specific activation of the PI3K-AKT pathway mediates a sex-dimorphic storage of glycogen in the pre-Sertoli cell lineage

Several growth factors essential for testis differentiation, such as fibroblast growth factor 9 (FGF9), platelet derived growth factor (PDGF) and insulin growth factor (IGF), are known to activate two major signaling pathways: the extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K)/AKT pathway. To analyze a possible role of the PI3K-AKT pathway in sex-dimorphic glycogen accumulation in developing gonads, we examined the effects of the PI3K inhibitor, LY294002, on glycogen accumulation in 10-11ts genital ridges in vitro (Fig. 8; Table 1). It was shown that the addition of LY294002 (15 μM) to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (Fig. 8C). XY genital ridges treated with 5 μM of LY294002 exhibited a partial inhibition of glycogen deposition, especially in the surface area of the XY gonadal explants (not shown). In all XY explants treated with 25 μM of LY294002, no PAS-positive cells were detected in their gonadal area (six out of six), suggesting that LY294002 dose-dependently inhibited glycogen accumulation in developing XY gonads. By contrast, the MEK (MAPK/ERK) inhibitor PD98059 did not have any obvious effect on glycogen accumulation in XY explants even at a concentration of 50 μM (Fig. 8B), despite the fact that administration of PD98059

drastically reduced the phosphorylation levels of the MEK substrate, ERK, in these explants (see Fig. S3 in supplementary material). Moreover, no appreciable difference was detected in XX explants in which no PAS-positive cells were found in the gonadal area of explants treated with LY294002 or PD98059 (Fig. 8D-F). These findings clearly suggest a possible involvement of the PI3K-AKT pathway in glycogen accumulation in developing XY gonads. To confirm whether or not the PI3K inhibitor LY294002 represses phosphorylation of its substrate AKT in gonads, we examined the phosphorylation level of XY and XX genital ridges by anti-phospho-AKT (active form of AKT) staining (Fig. 9). In the 48-hour control culture, anti-phospho-AKT immunohistochemical reactions were frequently observed in the gonadal area of XY explants (Fig. 9A). However, positive signals were only weakly detected in the gonadal area of XX explants, even though some positive signals were detected in their mesonephric regions. Moreover, the addition of the PI3K inhibitor LY294002 clearly reduced the intensity of anti-phospho-AKT staining in XY explants (XY+LY in Fig. 9A). Immunoblot analysis also confirmed that AKT was phosphorylated at a higher level in the XY explants than in XX explants in 24-hour control cultures of the genital ridges (cont in Fig. 9B). Moreover, in both XY and XX genital ridges, phosphorylation levels were repressed by the administration of LY294002 (+LY in Fig. 9B). By using anti-phospho-AKT staining of transverse sections of 11.5-dpc embryos, anti-phospho-AKT reactions were found in the ventral region of neural tubes and dorsal root ganglion at similar levels in both sexes, but their reactions in the gonadal region were higher in XY than in XX embryos in vivo (Fig. 9C). Anti-phospho-AKT reactions in XY gonads, both in vivo and in vitro, were found in somatic cells located near germ cells, i.e. presumptive Sertoli cells (Fig. 9A,C, right panels).

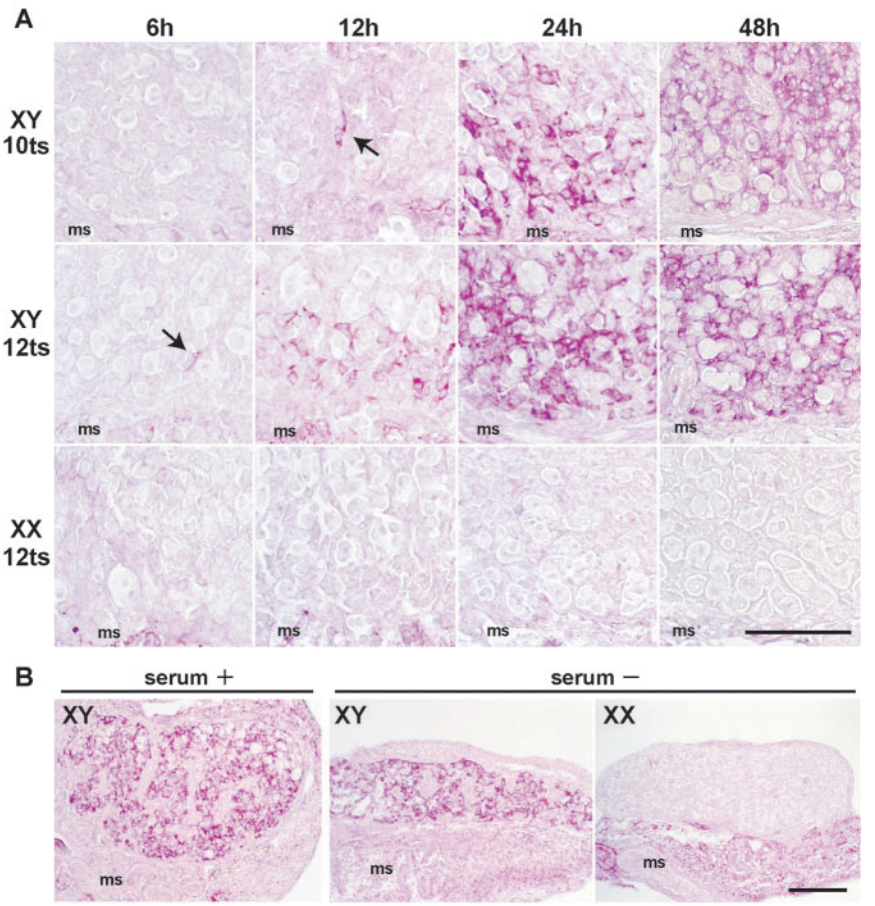

Fig. 6. Genital ridge organ cultures showing the time course of testis-specific glycogen accumulation (A) and testis-specific induction even in serum withdrawal (B). (A) Sagittal sections of genital ridges cultured with 10% horse serum-DMEM for 6, 12, 24 and 48 hours and stained with PAS. In XY explants isolated at 12 ts, PAS-positive cells are detected after being cultured for 6 hours (arrow in XY 12ts), and then rapidly increased in the gonadal area near germ cells from 12 to 24 hours. In XY genital ridges isolated at 10 ts, PAS-positive cells are induced in the gonadal region at 12-24 hours (arrow in XY 10 ts). By contrast, no PAS-positive cells are detected in the gonadal region of XX genital ridges even after being cultured for 48 hours (XX 12 ts). (B) Sagittal sections of XY (left and middle) and XX (right) genital ridges (12 ts) after 48-hour culture in medium with or without 10% horse serum and stained with PAS. Despite severe growth defects in the genital ridges, PAS-positive cells are found to be induced in the gonadal area of the XY, but not XX, genital ridge, suggesting no appreciable effect of serum withdrawal on sex-dimorphic glycogen deposition in vitro. ms, mesonephros. Bar, 50 μm.

Discussion

In mouse sex differentiation, *Sry* expression is first detected in the central region of XY gonads at around 12 ts (11.0 dpc), with its expression subsequently expanding to the entire gonadal area from 14 to 15 ts in a center-to-pole wave (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). The present study clearly demonstrates that glycogen accumulation starts to occur in pre-Sertoli cells in developing XY gonads from around 14 ts in a center-to-pole pattern similar to the *Sry* expression profile, suggesting a delay of only 2 ts (approximately 4 hours) after the onset of *Sry* expression. Moreover, the timing of the onset of accumulation is quite similar to that of testis-specific expression of SOX9, one of the first factors downstream of *Sry*. Therefore, these data indicate that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* action. Moreover, the present study shows that testis-specific glycogen deposition can be induced even in serum-free conditions and in a culture of gonadal explants without adjacent mesonephros, which suggests that sex-dimorphic glycogen deposition in developing gonads is tissue-autonomous. This is clearly in contrast to the essential roles of the gonad-mesonephros interaction and exogenous serum factors in testis-specific *Sox9* expression and/or maintenance in pre-Sertoli cells in vitro (Taketo and Koide, 1981; Tilmann and Capel, 1999;

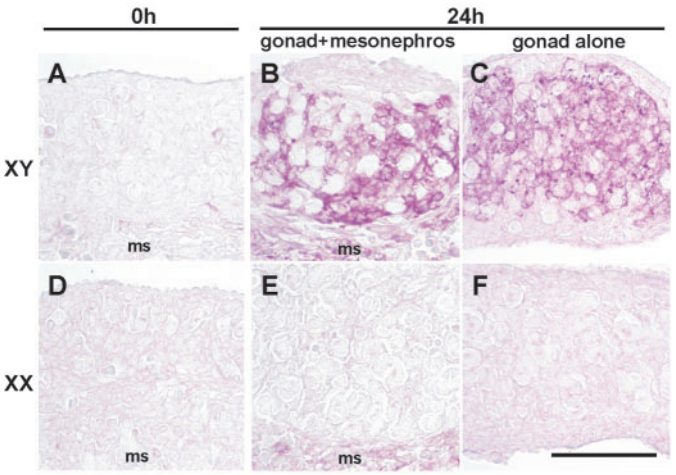

Fig. 7. Testis-specific glycogen accumulation does not require adjacent mesonephros in vitro. Sagittal sections of genital ridges (gonad + mesonephros; A,B,D,E) and genital ridges without adjacent mesonephros (gonad alone; C,F) isolated at 14 ts. PAS staining. Plates show the genital ridge after being cultured for 0 (A,D) or 24 (B,C,E,F) hours. In the 24-hour cultures, glycogen accumulation is induced in XY explants (A,B), but not in XX explants (D,E), of genital ridges. Similar to the pattern of glycogen accumulation in genital ridge cultures, PAS-positive cells are observed in XY explants without mesonephros (C), whereas no PAS-positive cells are detected in XX explants without mesonephros (F). ms, mesonephros. Bar, 50 μm.

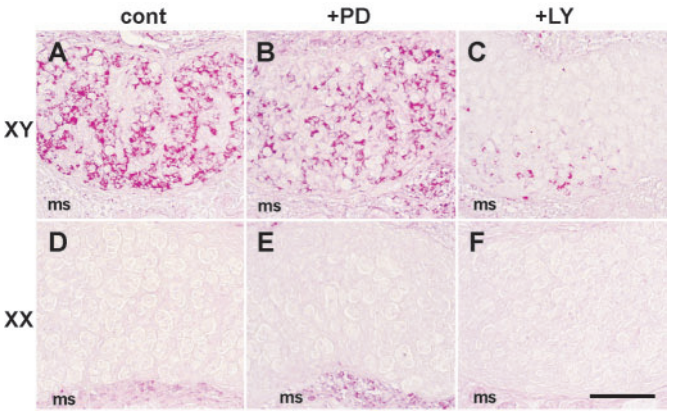

Fig. 8. Inhibition of PI3K inhibitor (+LY), but not MEK inhibitor (+PD), on testis-specific glycogen accumulation in developing XY genital ridges in vitro. Sagittal sections of genital ridges (10-11 ts) cultured in the absence (A,D) or presence of MEK inhibitor (50 μM, PD98059; B,E) or PI3K inhibitor (15 μM; LY294002; C,F) with 10% horse serum-DMEM for 48 hours and stained with PAS. In XY explants, the addition of LY294002 to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (C). By contrast, the MEK inhibitor PD98059 did not exert any obvious effect on glycogen accumulation in XY explants (B). Moreover, no appreciable glycogen accumulation is observed in XX genital ridges treated with these inhibitors (D-F). ms, mesonephros. Bar, 50 μm.

Puglianiello et al., 2004). This further suggests the possibility that glycogen accumulation in developing XY gonads is induced via a distinct pathway from *Sox9* regulation by SRY or that it occurs earlier than testis-specific *Sox9* expression in the sex-determining cascade.

The present in vitro analyses also demonstrate that the sex-dimorphic storage of glycogen granules in pre-Sertoli cells is mediated via the PI3K-AKT pathway, which is one of the major signaling pathways downstream of tyrosine kinase receptors for many polypeptide growth factors, including PDGF, FGF and insulin/IGFs (reviewed by Alessi and Cohen, 1998). This suggests that one or more of these growth factors are involved in testis-specific glycogenesis in pre-Sertoli cells. To date, it has been assumed that several growth factors are downstream of *Sry* and are important in testis differentiation. For example, the XY gonads of Pdgf-α receptor (*Pdgfra*)-knockout mice display disruptions in the organization of vasculature and in the partitioning of interstitial and testicular cord compartments (Brennan et al., 2003). In addition, studies have shown that *Fgf-9* is involved in testis-specific mesonephric migration and cell proliferation during testis differentiation (Colvin et al., 2001; Schmahl et al., 2004). By contrast, inhibition by the PI3K inhibitor LY294002 drastically disrupts cord formation and testis-specific mesonephric migration in vitro (Uzumcu et al., 2002). Although mesonephric migration does not make an appreciable contribution to sex-dimorphic glycogen deposition, these findings suggest that these growth factors induce glycogen accumulation in pre-Sertoli cells by activating the PI3K-AKT pathway in an autocrine and/or paracrine manner.

Most interestingly, Nef et al. have shown that insulin/IGF signaling is required for testis differentiation in mice (Nef et al., 2003). They demonstrated that XY mice with mutations for all three insulin receptor family members (*Ir*, *Igf1r* and *Irr*) developed ovaries and showed a completely female phenotype. Because it has been shown that insulin/IGF signaling generally stimulates glucose metabolism in target organs via the PI3K-AKT pathway (Saltiel and Kahn, 2001; Pirola et al., 2004), insulin/IGF is a strong candidate factor to directly regulate testis-specific glycogenesis in pre-Sertoli cells downstream of *Sry*. In fact, our preliminary in vitro experiments using the insulin/IGF inhibitor AG1024 (Calbiochem; 20 μM) and the PDGF inhibitor AG1295 (Calbiochem; 50 μM) showed that the insulin/IGF inhibitor AG1024, but not the PDGF inhibitor AG1295, significantly inhibited testis-specific glycogen accumulation in developing XY gonads, despite a similar level of reduction in phopho-AKT expression by both inhibitors (S.M. and Y.K., unpublished). However, Nef et al., also showed that the level of *Sry* expression was severely reduced in XY mice with mutations for all three insulin receptor family members, which suggests a possible role for insulin signaling upstream of *Sry* rather than a role in its downstream cascade (Nef et al., 2003). Therefore, further studies to identify growth factors that induce *Sry*-dependent glycogen accumulation and to determine how *Sry* activates the PI3K-AKT pathway in pre-Sertoli cells are warranted.

Glycogen accumulation is transiently observed in several tissues and cell types in various aspects of development. In the chick, for example, primordial germ cells contain an abundance of glycogen granules in their cytoplasm during the migratory phase, but they rapidly disappear shortly after entering the gonads (Clawson and Domm, 1963). The precursor cells of osteoblasts possess an accumulation of

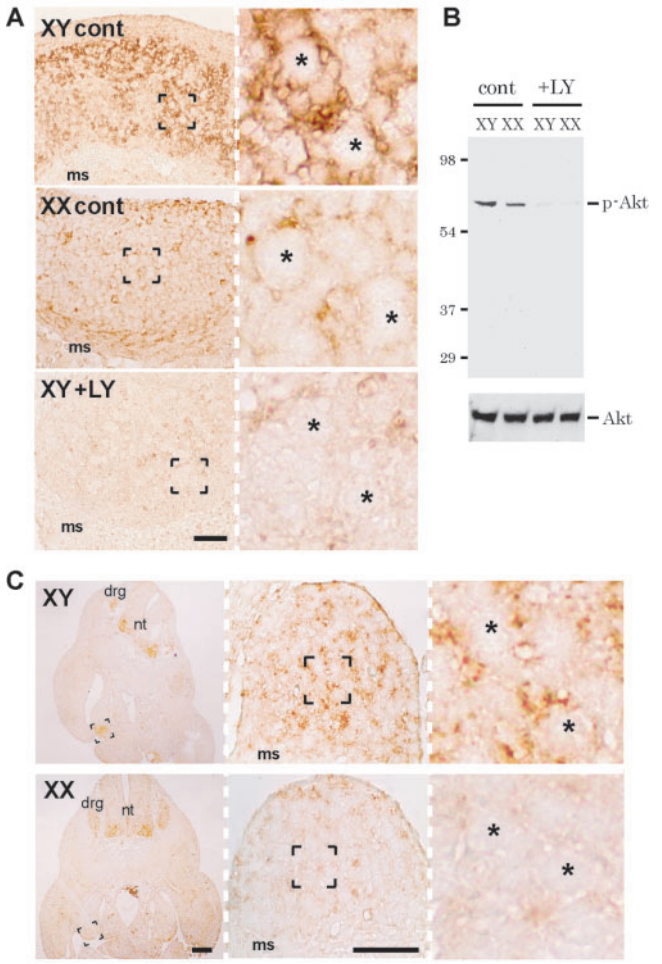

Fig. 9. Phosphorylation levels of AKT (a downstream effector of PI3K) in developing genital ridges in vitro and in vivo. (A) Sagittal sections of genital ridges (10-11 ts) cultured in the absence (cont) or presence of PI3K inhibitor (15 μM; LY294002; +LY) for 48 hours, and stained with anti-phospho-AKT. Positive signals for anti-phospho-AKT staining are detected in gonadal somatic cells located near germ cells, with a strong signal detected in the XY explant (XY cont) but only a weak signal in the XX explant (XX cont). Anti-phospho-AKT signals are clearly reduced in XY explants cultured in the presence of the PI3K inhibitor (XY + LY). (B) Immunoblot analyses of phosphorylation levels of AKT in XY and XX genital ridges (10-11 ts) cultured for 24 hours in the presence or absence of LY294002. In control XY explants, the AKT phosphorylation level is higher than that in XX control explants, and the level is clearly reduced by addition of LY294002 both in XY and XX explants. Repeat experiments were performed three times, and similar results were obtained each time. (C) Transverse sections of 11.5-dpc embryos (18 ts) and stained with anti-phospho-AKT. Anti-phospho-AKT reactions are found in the ventral region of neural tubes and dorsal root ganglion at similar levels in both sexes, but the reactions in the gonadal region are higher in XY than in XX embryos in vivo. These positive reactions in XY gonads are found in somatic cells near germ cells (asterisks). In plates A and C, right panels show higher magnification images, indicated by the boxed area in the corresponding left panel. asterisk, germ cell; drg, dorsal root ganglion; ms, mesonephros; nt, neural tube. Bar, 50 μm.

glycogen during fetal (Cabrini, 1961; Scott and Glimcher, 1971) and experimentally induced bone (Decker et al., 1995)

Table 1. Effects of MEK inhibitor, PD98059, and PI3K inhibitor, LY294002, on testis-specific glycogen accumulation in developing gonads in vitro*

Inhibitor	Sex	Glycogen accumulation in gonadal area† ++	+	+/–	–	Total number of explants
Control	XY	32	0	0	0	32
	XX	0	0	0	17	17
PD98059	XY	5	2	0	0	7
	XX	0	0	0	5	5
LY294002	XY	0	4	8	13	25
	XX	0	0	0	12	12

*Effects of each inhibitor (PD98059, 50 μM; LY294002, 15 μM) on glycogen accumulation were examined by a histochemical analysis of PAS reaction in a 48 hour culture of genital ridges isolated at 9-11 ts.

†Number of explants showing (1) no PAS-positive cells (–), (2) some PAS-positive cells detected in parts of gonadal area (+/–), or (3) PAS-positive cells detected throughout whole gonadal area, but the number of cells or PAS reactivity was reduced (+) when compared with those in control XY explants (++).

formation, and the loss of glycogen in differentiated osteoblasts is closely correlated to calcification (Harris, 1932). During tooth morphogenesis in mice, Ohshima et al. have suggested that glycogen deposition in the enamel organ cells from the cap to early bell stages (E14-E15) is associated with the active secretion of glycosaminoglycan components into extracellular spaces and the formation of the stellate reticulum (Ohshima et al., 1999). These reports clearly suggest that cell-type-specific glycogen depositions are closely associated with cell state, such as active migration, protein production, and secretion during tissue morphogenesis and differentiation. The testis differentiation is generally accompanied by dynamic morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian differentiation, and the onset of folliculogenesis occurs shortly before birth (18.0 dpc in mice). It is, therefore, reasonable to conclude that the preservation of energy stock as glycogen granules in differentiating pre-Sertoli cells reflects the active cell state associated with dynamic testis morphogenesis and the active production of testis-specific growth factors and structural components. This is clearly consistent with our previous observation that showed a rapid reduction in glycogen depositions in Sertoli cells after 12.5 dpc (Kanai et al., 1989).

In conclusion, the present study is the first to demonstrate that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry*. The present findings also illustrate that, immediately after the onset of *Sry* expression, extracellular signaling factors may promote glycogen accumulation in pre-Sertoli cells via the PI3K-AKT pathway. Previous ultrastructural studies of differentiating fetal Sertoli cells in various mammalian species generally exhibited an abundance of not only glycogen but also lipid droplets (another important energy source) and elongated, plemorphic mitochondria (energy-producing organelle) in their cytoplasm (reviewed by Gondos, 1977). Therefore, these data clearly raise an interesting hypothesis of a potential link between *Sry* action and sex-dimorphic energy metabolism in mammalian gonadal sex determination. Moreover, it would be interesting to know

whether or not such sex-dimorphic energy metabolism in gonadal sex differentiation is a conserved event in other vertebrate and invertebrate species.

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